Inhibition of insulin amyloid formation by small stress molecules

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Abstract Amyloidogenic proteins undergo an alternative folding pathway under stressful conditions leading to formation of fibrils having cross β-sheet structure, which is the hallmark of many neurodegenerative diseases. As a means of surviving against external stress, on the other hand, many microorganisms accumulate small stress molecules to prevent abnormal protein folding and to contribute to protein stability, which hints at the efficacy of the solutes against amyloid formation. The current work demonstrates the effectiveness of small stress molecules such as ectoine, betaine, trehalose, and citrulline on inhibition of insulin amyloid formation in vitro. The inhibitory effects were analyzed by thioflavin T-induced fluorescence, circular dichroism, and atomic force microscopy. This report suggests that naturally occurring small molecules may serve a function that is typically fulfilled by protein chaperones, and it provides a hint for designing inhibitors against amyloid formation associated with neurodegenerative disorders.

Key words: Amyloid formation; Insulin; Inhibitor; Small stress molecule

1. Introduction

A number of structurally different proteins are now known to form amyloid fibrils when subjected to stress conditions like high temperature and extreme pH [1–4]. These environmental stresses trigger off an alternative folding pathway for amyloidogenic proteins, leading to partial unfolding of proteins followed by formation of amyloid having cross β-sheet structure [5,6]. The formation of amyloid aggregates and their deposition in tissues is the pathological hallmark of many neurodegenerative diseases such as Alzheimer's and Parkinson's disease, mad cow disease, and others [4,5]. While it is still unresolved how normal and soluble proteins assemble into fibrillar and insoluble amyloid aggregates under certain conditions [7], abnormal accumulation of amyloid oligomers, protofibrils, or fibrils is considered to be one of the major contributing factors to neurodegeneration [8–13]. Due to the possible relation of amyloid to pathology, many efforts are underway to screen compounds that interfere with amyloid formation, and a few of those inhibitors are currently undergoing clinical trials as potential drugs to treat neurodegenerative disorders [4,10,11,14,15].

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While environmental stress is a contributing factor in protein denaturation and aggregation [16,17], some life forms in nature have successfully adapted to specific types of stresses [18,19]. For example, hyperthermophiles, which are microorganisms capable of growing at temperatures as high as 113°C, accumulate unusual molecules called organic solutes or small stress molecules as a means of surviving against thermal stress [20–22]. *Pyrococcus aerophilum* (T_{opt} = 100°C) and *Thermus thermophilus* (T_{opt} = 70°C) were reported to accumulate small solutes such as trehalose and betaine in high concentrations against heat stress [20,21]. Increasing evidence shows that small stress molecules can contribute to thermostability of enzymes, possessing the ability to maintain protein functionality under extreme environments by stabilizing the native conformation of proteins [22–24]. Presence of small stress molecules like ectoine, betaine, and trehalose had proved highly effective in preserving enzymatic activities against heat treatment, freeze-thawing, and freeze-drying. For example, these solutes elevated the temperatures at which 50% loss of lactic dehydrogenase and phosphofructokinase activities was observed up to 14°C [25,26]. Citrulline is found in wild watermelon leaves and acts as a free radical scavenger, contributing to the high drought tolerance of the plant [27]. Those works show the protective nature of small stress molecules for maintaining protein stability and activity suggesting the efficacy of the stress molecules against amyloid formation, which has been rarely studied so far.

In the present work, we report that the small stress molecules strongly inhibit amyloid formation of insulin in vitro. Insulin amyloid formation, a characteristic of injection-localized amyloidosis, occurs through nucleation, elongation, and saturation stages like other amyloidogenic proteins, and its rate is highly accelerated under stressful conditions such as high temperature, extreme pH, and high shear stress [28–32]. Our finding can be useful for designing inhibitors that work against amyloid formation.

2. Materials and methods

2.1. In vitro insulin amyloid formation

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solution of bovine insulin was made at 2 mg/ml in glycine buffer (pH 2, 20 mM). It was then diluted with glycine buffer to 1 mg/ml for control experiments and with solutions containing stress molecules in glycine buffer for inhibition studies. Incubation was done in sealed glass vials (1.8 ml) to prevent any possible evaporation during incubation. In our preliminary experiments, insulin solution was incubated at 37°C, 44°C, and 50°C to observe the effect of heat stress on amyloid formation. Application of heat stress shortened the lag time for insulin amyloid formation and increased the maximal value of thioflavin T (ThT) fluorescence at stationary phase.

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In this work, 50°C was selected as the incubation temperature for control experiments, where lag time and apparent rate constant (k_{app}) were 12.3 h and 2.47 h^{-1}, respectively. For observing the effect of preformed nuclei, seed solution was made by incubating 1 mg/ml insulin solution (pH 2, 20 mM) at 50°C for 18 h. This pre-incubated solution was added to freshly prepared 1 mg/ml insulin solution (pH 2, 20 mM) to make 1% of total solution volume.

2.2. ThT fluorescence measurement

ThT solution (50 μM, 1.5 ml) was mixed with 5 μl samples in a quartz cuvette. All fluorescence measurements were carried out with a spectrofluorophotometer (Model RF5301, Shimadzu, Japan) according to [33]. The excitation wavelength was set at 450 nm with emission measured at 482 nm with excitation and emission slit widths at 5 nm each. Each reading was done in triplicate and the average was used for data analysis.

2.3. Circular dichroism (CD) measurement

The samples (20 μl) were dialyzed before CD spectrum measurement to avoid possible interference with stress molecules. Samples were placed in mini-dialysis units from Fisher Scientific (Rockford, IL, USA), suspended in glycine buffer (pH 2, 20 mM), and left for 12 h. The resultant volume was then diluted to 300 μl in a cell with a 0.1 mm path length for use in CD spectrum measurement. Interference with ectoine and citrulline could not be removed even with the dialysis treatment. CD spectra were measured on a spectropolarimeter (Model J-710, Jasco, Japan) from 190 nm to 260 nm at a scan speed of 50 nm/min and a resolution of 0.5 nm according to Bouchard et al. [29].

2.4. Atomic force microscopy (AFM)

AFM was used to visualize amyloid fibrils as per the method described by Stine et al. [34]. An aliquot of 5 μl from the incubated solution was placed on freshly cleaved mica at room temperature for a few seconds. Samples were diluted twice with 50 μl of deionized water followed by drying with nitrogen gas. AFM tips were used at a resonant frequency of 306–444 kHz for imaging fibrils with a Nanoprobe III scanning probe workstation (Digital Instruments, CA, USA). Each image was acquired in tapping mode under ambient conditions at a scan frequency of 1–2 Hz. Two different samples were analyzed in each case and at least five spots with an area of 2×2 μm were scanned. The samples were used without dilution to maintain the same concentration of fibrils as present in the originally incubated volume. Representative images were selected for comparative studies.

2.5. Evaluation of amyloid formation kinetics

The growth of amyloid structure, as depicted by the increase in ThT fluorescence, followed a typical sigmoidal curve, having an initial lag phase with no increase of fluorescence, followed by elongation and then the saturation phase. The fluorescence data obtained were fitted with a four-parameter sigmoidal curve using Sigma Plot v. 8.02 (SSPS, Chicago, IL, USA) as per the following equation:

$$y = y_o + \frac{a}{1 + e^{-\left(\frac{x - x_o}{b}\right)}}$$

where y is the fluorescence at time x, y_o is the initial fluorescence value, x_o is the time when fluorescence reaches 50% of its maximum value, and a is the maximal fluorescence at stationary phase. Kinetic constants were calculated as apparent rate constant ($k_{app} = 1/b$) and lag time ($x_o - 2b$).

3. Results and discussion

Ectoine, trehalose, and citrulline completely suppressed insulin amyloid formation at 300 mM as indicated by absence of ThT fluorescence even after 40 h of incubation (Fig. 1). Betaine also showed an inhibitory effect at 300 mM, reducing stationary ThT fluorescence to one third of the control value. In contrast, maltose and sucrose, which are disaccharides having structures similar to that of trehalose, did not suppress amyloid formation at the same concentration (data not shown). According to AFM images, the control sample contained a high density of typical unbranched, insulin amyloid fibrils (Fig. 2A), while fibrils were rarely found in the samples with trehalose (Fig. 2C), betaine (Fig. 2D), ectoine (Fig. 2E), and citrulline (Fig. 2F). However, high fibril densities were observed in the presence of maltose, which indicates a lack of inhibitor action (Fig. 2B). Small stress molecules were found to have inhibitory effects at lower concentrations as listed in Table 1. The apparent rate constants for all incubations in the presence of stress molecules were found to be significantly lower than control value.

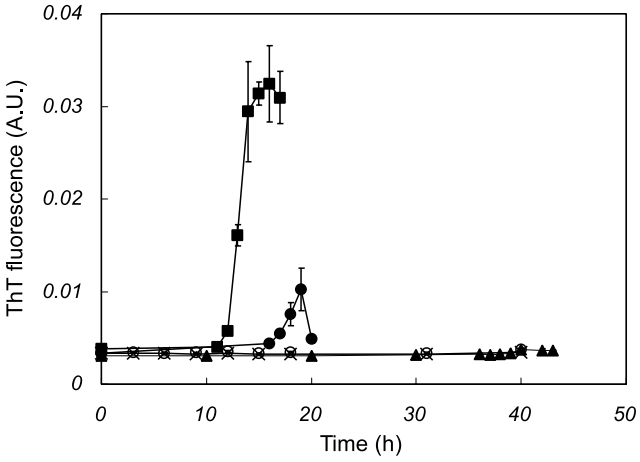

Fig. 1. Effect of small stress molecules on insulin amyloid formation. Control (■), trehalose (▲), betaine (●), ectoine (×) and citrulline (○). All stress molecules were at 300 mM concentration. Data shown are ThT time profiles for insulin (1 mg/ml, pH 2) amyloid formation incubated at 50°C. Error bar represents the standard deviation in triplicate measurements.

According to CD analysis, native insulin exhibited two minima at 208 nm and 222 nm, which were replaced by a single minimum at 216 nm with the progress of incubation at 50°C (Fig. 3A). This observation is in agreement with the report by Bouchard et al. [29]. The CD spectra for insulin solution containing betaine and trehalose showed a single minimum between 208 and 216 nm indicating decreased amyloid formation in the presence of these solutes (Fig. 3A). Trehalose showed a greater inhibitory effect compared to betaine with CD spectrum closer to that of native insulin. The CD spectra of trehalose (Fig. 3B) show that higher amounts of native insulin were present with increased trehalose concentrations as indicated by higher values of negative ellipticity at 208 nm. In case of ectoine and citrulline, their own interference with insulin CD spectra could not be removed even with dialysis treatment (data not shown).

Amyloid fibril formation is known to occur through nucleation and elongation as intermediate steps [30]. In this work, in order to figure out which of these two stages is actually the point of inhibition by small stress molecules, insulin amyloid formation was carried out in the presence of pre-incubated solution (1% v/v). Addition of the pre-incubated solution could supply amyloid seed/nuclei to the fresh insulin solution, thereby leaving only the elongation stage for inhibitor action by small stress molecules. In seeded solution, insulin amyloid formation occurred with negligible lag phase (Fig. 4). According to the ThT fluorescence profiles, inhibitory effects by trehalose, betaine, and citrulline were significantly reduced in the presence of seeds except for ectoine. The decreased inhibitory effect was also confirmed by AFM analysis, where high den-

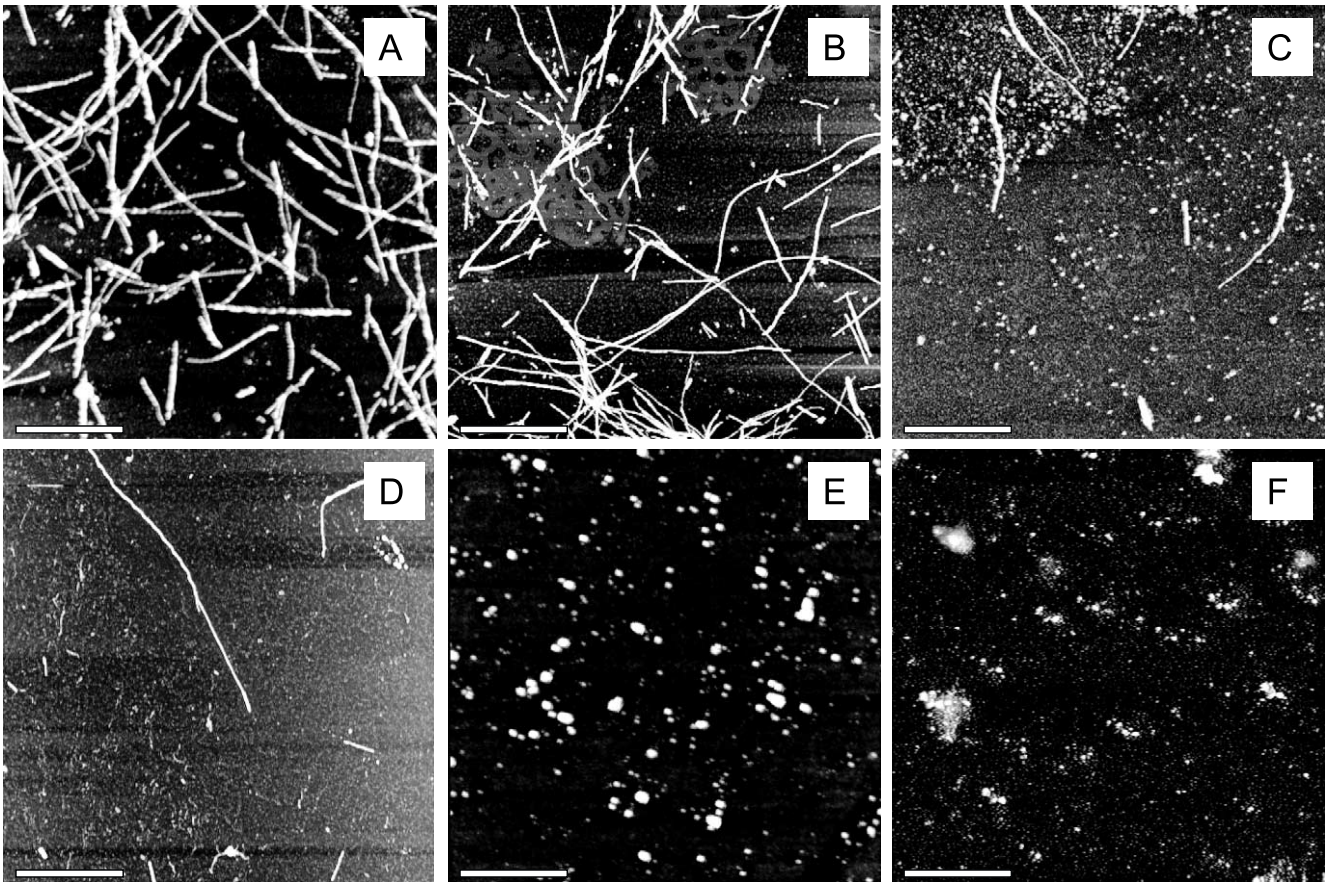

Fig. 2. AFM images of samples incubated with small stress molecules. A: Control. B: Maltose. C: Trehalose. D: Betaine. E: Ectoine. F: Citrulline. All the small stress molecules were at 300 mM. The images were taken at saturation phase in tapping mode. Scale bar represents 500 nm.

sity insulin fibrils were observed even with the small solutes (data not shown). This result suggests that the inhibitory effect of trehalose, betaine, and citrulline is partially nucleation-specific, whereby formation of amyloid oligomers or nuclei is prevented.

The observed phenomenon was hypothesized to be the combined effect of increased surface tension of solution coupled with preferential hydration of insulin monomers by the presence of small stress molecules. Small stress molecules were reported to increase the surface tension of aqueous solutions, while they were preferentially excluded from the vicinity of protein monomers making the proteins hydrated with solvent molecules [23]. Unfolding of protein monomers increases the surface area available to protein–solvent interaction [35,36]. This process becomes energetically unfavorable in the presence of small stress molecules due to the positive free energy change caused by the increased surface tension of solution. Thus, according to our hypothesis, preferential hydration in conjunction with increased surface tension forces insulin monomers to retain a compact configuration in order to minimize energy of solvent–protein interaction. This mechanism works against hydrophobic interaction of partially unfolded insulin monomers, one of the major driving forces for amyloid formation [30,37,38]. The amyloid formation process is believed not to be dependent on 'specific' protein–solvent pairs [36], thereby increasing the chances of efficacy of small stress molecules against fibrillization of other amyloidogenic proteins. Further verification of this proposed mechanism is awaited. It is noteworthy that molecular chaperones have been reported to possess functional similarity to the small stress molecules by restoring native protein structure with their action against hydrophobic interactions [39]. Heat shock

Table 1
Dependence of apparent rate constant (k_{app}) of amyloid formation on the concentration of small stress molecules

Solute concentration (M)	k_{app} observed in the presence of solute (h^{-1})[a]			
	Ectoine	Citrulline	Betaine	Trehalose
0.05	0.64	0.96	1.29	1.27
0.10	0.88	0.94	1.98	1.57
0.20	0.84	0.44	1.29	0.68
0.30	nd[b]	nd[b]	1.50	0.67

[a]In the absence of solute, k_{app} was found to be 2.47 h^{-1}.
[b]Not determined due to negligible increase of ThT fluorescence for the time period up to 45 h.

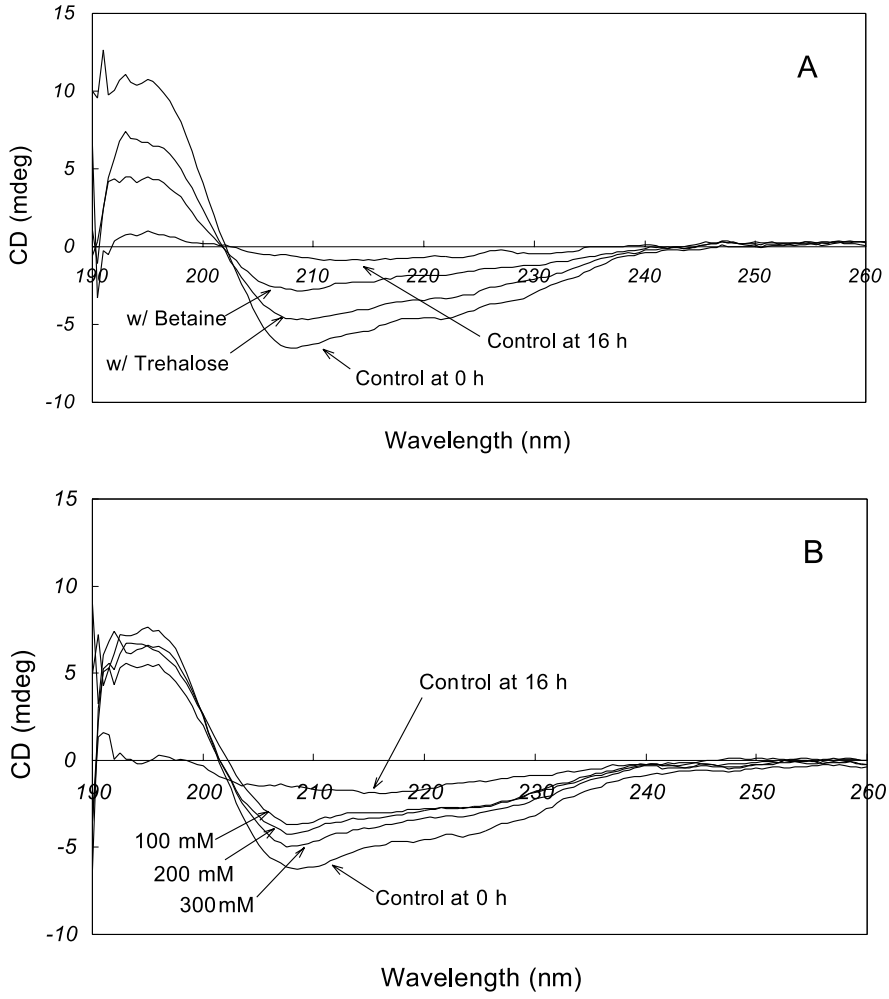

Fig. 3. Near-UV CD spectra for insulin amyloid inhibition in the presence of small stress molecules. A: Effect of trehalose and betaine (300 mM) on insulin amyloid formation. B: Concentration-dependent inhibitory effect of trehalose on amyloid formation. All spectra were measured at saturation phase at ambient temperature.

proteins such as sHsp27 and Hsp70 suppressed Aβ1–42 amyloid formation and α-synuclein toxicity, respectively [40,41].

In this work, small stress molecules, i.e. ectoine, betaine, trehalose, and citrulline, were found to inhibit insulin amyloid formation. Previous works in the literature prove that the stress molecules interact with and stabilize the hydrophilic groups on protein moieties by solvent hydration while the hydrophobic groups remain on the inside of proteins. This stabilization process works well against insulin amyloid formation driven by hydrophobic interactions between partially unfolded protein monomers. The probability of success of small stress molecules against amyloid formation is further strengthened by a recent report on the efficacy of trehalose against Huntington disease [42], which was published at the time of submitting this article. According to the report, trehalose suppressed the formation of polyglutamine aggregates with strongest inhibitory effect among various disaccharides when administered orally as a 2% solution to transgenic mice. The current work, supported by [42], hints at the efficacy of small stress molecules as lead compounds for designing inhibitors against amyloid formation associated with neurodegenerative disorders.

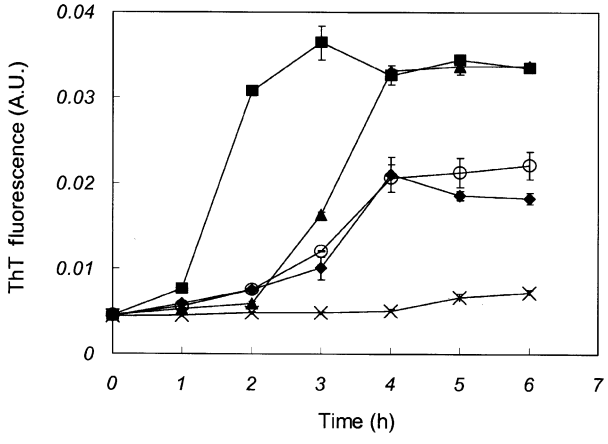

Fig. 4. Effect of small stress molecules on amyloid formation in insulin solution that contains amyloid seeds (1% v/v). Control (■), trehalose (▲), betaine (●), ectoine (×) and citrulline (○). The amyloid seeds were prepared by pre-incubation for 18 h. Final concentrations of insulin and stress molecules in each vial were the same as in Fig. 1.

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